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Clostridium perfringens associated with dairy farm systems show diverse genotypes

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ABSTRACT

Clostridium perfringens is a bacterial species of importance to both public and animal health. Frequently found in food system environments, it presents a risk to food animal health such as dairy herds, and may cross contaminate associated ingredients or food products, with potential to cause sporadic and outbreaks of disease in human populations, including gastroenteric illness. In this study, we characterized *C. perfringens* isolated from bovine, caprine, and ovine dairy farm systems (n = 8, 11 and 4, respectively). Isolates were phenotypically screened for antimicrobial sensitivity profiling, and subjected to whole genome sequencing to elucidate related genetic markers, as well as examine virulence gene markers, mobile genetic elements, and other features. Both toxin type A and type D isolates were identified (78 % and 22 % of isolates, respectively), including 20 novel sequence types. Resistance to clindamycin was most prevalent among antibiotics screened (30 %), followed by erythromycin (13 %), then penicillin and tetracycline (4 %), although an additional 3 isolates were non-susceptible to tetracycline. Most isolates harboured plasmids, which mobilised virulence markers such as *etx*, *cpb2*, and resistance markers *tetA(P)*, *tetB(P)*, and *erm(Q)*, on conjugative plasmids. The presence of type D isolates on caprine farms emphasizes the need for control efforts to prevent infection and potential enterotoxemia. *Clostridium perfringens* enterotoxin (*cpe*) was not identified, suggesting lower risk of gastrointestinal illness from contaminated foods, the presence of other virulence and antimicrobial resistance markers suggests farm hygiene remains an important consideration to help ensure food safety of associated dairy foods produced.

1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic spore-forming bacterium, known as the causative agent of various intestinal diseases in humans and animals (Brynestad et al., 2001). Notably in humans, *C. perfringens* is the major causative agent of gas gangrene, a rapid destruction of tissues with production of gas (Hatheway, 1990). It is also an important agent of foodborne gastroenteritis (WHO, 2015). In animals, including sheep, goats, cattle, and poultry, *C. perfringens* can cause enterotoxemia and necrotic enteritis, among other diseases (Lebrun et al., 2010; Uzal et al., 2014). This opportunistic pathogen is commonly found in a variety of environments, including soils, food, freshwater sediments, sewage systems, and the normal intestinal flora of humans and animals (Matches et al., 1974; Mullié et al., 2002). As such, the bacterium is often widely distributed through dairy farm production systems, including soil associated with pastures, and water used for feeding or irrigation; with wider dissemination supported through fecal shedding of colonised animals, such as ruminants. Animals themselves represent a key cross-contamination route for raw milk, as well as other farm-associated niches, through teat or hide contamination, particularly where fecal material is associated. Although milk filters/socks can be used to capture gross debris from entering the bulk milk tank, these filters do not generally prevent transfer of microbes such as *C. perfringens,* Thus, milking and herd hygiene is critical to reducing or preventing cross-contamination of raw milk.

Strains of *C. perfringens* may be categorized into different toxin genotypes, based on the combination of specific toxins they may produce. Traditionally classified in to one of five toxin types (type A, B, C, D, or E); this has recently been updated to include two new genotypes, type F and type G (Rood et al., 2018). These toxins contribute to a variety of disease

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conditions, including dysentery, enterotoxaemia, necrotising enteritis, or gas gangrene, often displaying particular host specificities in relation to disease pathology and/or severity (Uzal et al., 2014). This host-specificity places a given host at higher threat from certain toxigenic subtypes, for example type A-associated necrotising enteritis in poultry, or type D-induced enterotoxemia of small ruminants (Uzal and Songer, 2008; Van Immerseel et al., 2004). All toxin types harbor the alpha toxin, CPA, encoded by the *cpa* (or *plc*) gene. Type D strains also produce the epsilon toxin, ETX, the product of the plasmid-borne *etx* gene. Although more commonly associated with disease in smaller ruminants like sheep and goats, it may also illicit enterotoxemia in larger cattle (Uzal et al., 2014). Type D strains may also carry the enterotoxin gene *cpe*, which is primarily associated with gastroenteric illness in humans; however, this toxin is not carried in type A strains. As such, CPE-producing strains are particularly high risk for sporadic incidence and/or outbreaks of foodborne illness in human populations.

Historically, the use of antibiotics as growth promoters for food producing animals was widespread globally; in recent years, however, a recognition of the growing problems surrounding antimicrobial resistance (AMR) and public health has led to tighter controls around antimicrobial stewardship, and a reduction in this practice (Ma et al., 2021). Antimicrobial treatment of *C. perfringens* infection is required for the more severe forms of illness; however, resistance has been observed against a variety of antimicrobial agents, such as bacitracin, imipenem, and tetracycline (Álvarez-Pérez et al., 2017; Slavić et al., 2011; Tansuphasiri et al., 2005). The prevalence of resistance, however, shows geographical variation.

Understanding the molecular ecology of *C. perfringens* on dairy farms is important to elucidate dissemination routes on farm, and help control exposure of associated food and food animals. To understand the associated risks to animal and public health, it is also essential to understand key characteristics of strains such as toxins, antimicrobial resistance markers, and mobile genetic elements that may mobilise their transfer through the *C. perfringens* population. In this study, we characterized *C. perfringens* isolated from dairy farm herds and associated environments, to examine the molecular ecology and antimicrobial sensitivity of the population. Dairy farms included bovine, caprine, or ovine herds, producing milk for fluid milk and/or cheese products. Interrogation of draft genomes was employed to elucidate the molecular markers relating to virulence and AMR, and examine the genomic context of these to indicate potential dissemination through the population.

2. Materials and methods

2.1. Bacterial isolates in this study

This study characterized 23*C. perfringens* isolates collected across 7 dairy farms in Victoria, Australia (McAuley et al., 2014). This included bovine ($n = 3$), caprine ($n = 3$) and ovine ($n = 1$) farms, and from samples including soil ($n = 6$), faeces ($n = 8$), feed ($n = 4$), milk ($n = 3$) and milk filters ($n = 2$; Table 1).

2.2. Antimicrobial sensitivity profiling

The minimum inhibitory concentrations (MICs) of isolates to a panel of 7 antimicrobials was performed using gradient M.I.C.Evaluator (Oxoid, Hampshire, UK) and Etest strips (Biomerieux, Marcy-l'Étoile, France). The panel included: chloramphenicol, metronidazole, penicillin G, rifampicin, and tetracycline (0.002–32 mg/L); clindamycin (0.016–256 mg/L); and erythromycin(0.015–256 mg/L). Screening was performed as per manufacturers instruction. Breakpoints were taken from: EUCAST recommendations for *C. perfringens* for clindamycin, metronidazole, and penicillin (The European Committee on Antimicrobial Susceptibility Testing, 2022); CLSI for chloramphenicol and tetracycline (CLSI, 2014); with those of both erythromycin and rifampicin taken from Álvarez-Pérez et al. (2016).

2.3. Genome assembly and annotation

Genomic DNA was extracted using the QIAGEN DNeasy kit (QIAGEN, Germany), using pre-treatment in enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2 % Triton X-100, 20 mg/L lysozyme), as recommended. Sample quality was confirmed using a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA) to confirm 260:280 nm and 260:230 nm ratios between 1.8 and 2.0. Library preparation using genomic DNA of isolates was performed using the Nextera XT library prep kit (Illumina, San Diego, CA). Raw read sequences were then generated using 250-bp paired end sequencing on the MiSeq platform (Illumina). The raw read quality was assessed with FastQC (version 0.11.8). These raw reads were subsequently processed to remove adapter sequences and low quality reads using Trimmomatic software v0.22 (Bolger et al., 2014). Draft genomes were assembled using SPAdes (Species Prediction and Diversity Estimation) software v2.5.1 based on

Table 1
Summary of the isolation details, and associated genomes, of strains in this study. The final three columns indicate presence/absence of at least one: plasmid, plasmid with a TCP conjugal transfer locus, and plasmid with a PCP conjugal transfer locus, respectively.

Isolate	Toxin type	Source	Farm herd	Farm	Year	Genome size (bp)	GC content (%)	Number of CDS	Plasmid	TCP	PCP
Cp13–001	A	Faeces	Bovine	Bov1	2013	3,422,224	28.3	3033	+	+	–
Cp14–001	A	Faeces	Bovine	Bov1	2014	3,530,157	28.3	3150	+	+	–
Cp13–003	A	Faeces	Bovine	Bov2	2013	3,061,807	28.4	2722	–	–	–
Cp13–007	A	Faeces	Bovine	Bov3	2013	3,525,066	28.0	3203	+	+	+
Cp14–004	A	Faeces	Bovine	Bov3	2014	3,266,164	28.3	2872	–	–	–
Cp14–016	A	Faeces	Caprine	Cap2	2014	3,532,289	28.1	3192	+	+	–
Cp13–014	A	Faeces	Ovine	Ov1	2013	3,330,542	28.2	2914	+	+	–
Cp14–011	A	Faeces	Ovine	Ov1	2014	3,202,418	28.4	2764	–	–	–
Cp13–011	D	Feed	Caprine	Cap1	2013	3,641,051	27.8	3284	+	+	–
Cp14–010	A	Feed	Caprine	Cap1	2014	3,653,505	28.1	3332	+	+	–
Cp13–019	A	Feed	Caprine	Cap2	2013	3,269,224	28.2	2817	–	–	–
Cp14–021	A	Feed	Caprine	Cap3	2014	3,090,393	28.3	2745	–	–	–
Cp14–007	D	Milk	Caprine	Cap1	2014	3,567,351	28.0	3241	+	+	–
Cp14–008	A	Milk	Caprine	Cap1	2014	3,323,440	28.2	2940	–	–	–
Cp14–014	D	Milk	Caprine	Cap2	2014	3,418,938	28.0	3038	+	+	–
Cp14–002	A	Milk Filter	Bovine	Bov1	2014	3,661,219	28.3	3217	+	+	–
Cp14–019	D	Milk Filter	Caprine	Cap2	2014	3,446,361	28.1	3051	+	+	–
Cp13–005	A	Soil	Bovine	Bov2	2013	3,307,783	28.3	2909	+	–	–
Cp14–006	A	Soil	Bovine	Bov3	2014	3,414,107	28.3	2984	+	+	–
Cp13–009	A	Soil	Caprine	Cap1	2013	3,586,893	27.9	3244	+	+	–
Cp13–017	D	Soil	Caprine	Cap2	2013	3,535,408	28.0	3160	+	+	–
Cp13–015	A	Soil	Ovine	Ov1	2013	3,488,526	28.1	3089	+	+	–
Cp14–013	A	Soil	Ovine	Ov1	2014	3,152,455	28.4	2674	–	–	–

an algorithm which employs multi-sized De Bruijn graphs with *k-mer* values of '21, 33, 55, 77' to construct the contiguous sequences (Bankevich et al., 2012). All draft genomes were annotated using the RAST online platform tool, and using Prokka algorithms (Aziz et al., 2008; Seemann, 2014).

2.4. Phylogenetic analyses

The isolates were classified into sequence types (STs) based on the established *C. perfringens* MLST subtyping scheme (Deguchi et al., 2009). MLST alleles were screened using the online PubMLST platform (Jolley et al., 2018), and compared to known ST scheme to match ST, and compare with similar strains. To further examine the genetic relatedness of isolates, a maximum likelihood comparison using RAxML analysis of annotated draft genome assemblies was performed (Stamatakis, 2014), in addition to phylogenetic clustering based on core genome single nucleotide polymorphisms, performed using the Snippy pipeline (Seemann, 2015). The *C. perfringens* Type strain ATCC 13124 was used as a reference in the Snippy analysis.

2.5. Genome screening for molecular markers, and comparative visualisation of sequence data

A strain BLAST database was created using the Geneious Prime software platform (Kearse et al., 2012). Additional databases were created comprising genes of interest relating to virulence, stress resistance, or other features such as mobile genetic elements, as detailed in Supplementary Table 1. Sequence alignments were performed using MAFFT program (Katoh et al., 2002). EasyFig software was utilized to visualise sequence alignment similarities, including transposon and phage alignments (Sullivan et al., 2011). The BLAST ring image generator (BRIG) platform was used to visualise BLAST comparisons using constructed pangenome references (Alikhan et al., 2011).

2.6. Pangenome analysis

Pan-genome analysis was performed utilising the Roary pipeline (Page et al., 2015), and RAxML maximum likelihood phylogenetic trees were constructed using these annotated assemblies (Stamatakis, 2014). Pangenome interrogation for phage insert regions was performed using the online PHASTER tool (Arndt et al., 2016). CRISPRFinder was utilized to identify confirmed and putative CRISPR regions (Grissa et al., 2007).

2.7. Draft sequence archiving

Draft genome sequences for isolates from this project have been deposited in the NCBI genome database, BioProject PRJNA772003, under the following accessions: Cp13-001, JAJCSE000000000; Cp13-003, JAJCSF000000000; Cp13-005, JAJCSG000000000; Cp13-007, JAJCSH000000000; Cp13-009, JAJCSI000000000; Cp13-011, JAJCSJ000000000; Cp13-014, JAJCSK000000000; Cp13-015, JAJCSL000000000; Cp13-017, JAJCSM000000000; Cp13-019, JAJCSN000000000; Cp14-001, JAJCSO000000000; Cp14-002, JAJCSP000000000; Cp14-004, JAJCSQ000000000; Cp14-006, JAJCSR000000000; Cp14-007, JAJCSS000000000; Cp14-008, JAJCST000000000; Cp14-010, JAJCSU000000000; Cp14-011, JAJCSV000000000; Cp14-013, JAJCSW000000000; Cp14-014, JAJCSX000000000; Cp14-016, JAJCSY000000000; Cp14-019, JAJCSZ000000000; Cp14-021, JAJCTA000000000.

3. Results

3.1. Overview of the genomes

An overview of the genome characteristics is provided in Table 1. This included 18 toxin type A isolates and 5 type D isolates. Genome size ranged from 3,061,807 bp (Cp13-003) up to 3,661,219 bp (Cp14-002). Isolates without plasmids were among those with the smallest genome sizes (all 7 of the isolates that did not contain plasmids were among the 8 smallest genomes). The number of coding sequences ranged from 2722 to 3217, and the GC content from 27.8 % to 28.4 %. The isolates shared a core genome of 1793 genes (20.8 %), from a total pangenome size of 8602 genes (Supplementary Fig. 1; Hadfield et al. (2017)). Considering the type A isolates alone, they shared a core genome of 1799 (23.0 %) from a pangenome totalling 7820 genes. The type D cohort showed greater genome homology, with a core genome of 2494 genes (56.7 %) out of a pangenome of 4398 genes.

3.2. Phylogenetic analyses

The *C. perfringens* MLST analysis was based on the PubMLST database records, which includes 553 isolates as of November 17th, 2021. Of the 23 isolates in this study, Cp14-013 was the only isolate with a previously described ST (ST291). All other isolates had novel STs; of these, 3 isolates shared the same ST (type D isolates Cp13-011, Cp14-014, and Cp14-019), with the others having their own unique ST (Fig. 1). An additional 38 isolates were included in the MLST analysis, including representatives of all 7 toxin types. With the exception of a single node containing 23 isolates (all *netF*-positive strains linked with cases of necrotizing enteritis in foals or haemorrhagic diarrhea in canines; Mehdizadeh Gohari et al. (2017)), strains showed a diverse molecular ecology, with few clonal complexes noted among represented STs. Type A isolates from this study, largely, did not cluster into defined subgroups (Fig. 2); although type D isolates showed greater sequence homology with respect to isolates from this study (3 isolates sharing a ST), more generally, the 6 type D isolates also showed diverse genetic lineage (Figs. 1 & 2).

3.3. Antimicrobial resistance

Among the antibiotics screened, all isolates were susceptible to chloramphenicol, metronidazole, and rifampicin (Table 2). The highest resistance prevalence noted was to clindamycin, comprising 30 % of isolates ($n = 7$). One isolate ($n = 1$, 4 %) was resistant to tetracycline (Cp14-001); an additional 3 isolates, (Cp13-001, Cp13-003, and Cp14-002), showed intermediate sensitivity to this antibiotic. A single isolate ($n = 1$, 4 %), Cp13-001, showed multidrug resistance to clindamycin, erythromycin, and penicillin, in addition to non-susceptibility to tetracycline. Two additional isolates were resistant to both clindamycin and erythromycin: Cp14-001 and Cp14-002; ($n = 2$, 9 %); the former being resistant to tetracycline, while the latter was non-susceptible to this antimicrobial. Overall, 8 isolates (35 %) showed resistance to at least 1 antibiotic. Most resistant isolates were type A (88 %); only a single type D isolate showed resistance to clindamycin. All three multidrug resistant isolates originated from a single farm (bovine farm A). Of the isolates demonstrating antimicrobial resistance, the majority were derived from fecal samples ($n = 5$, 63 %), with others from soil, milk or milk filter samples ($n = 1$ for each).

Resistance phenotypes could typically be correlated to associated genetic markers (Fig. 3): the tetracycline-resistant isolate Cp14-001 harboured both *tetA*(P) and *tetB*(P), as did the intermediate resistant isolates Cp13-001 and Cp14-002; Cp13-003, which showed intermediate resistance to tetracycline, but at a lower MIC to other non-susceptible isolates, harboured *tetA*(C) alone; isolates resistant to erythromycin and higher clindamycin resistance carried *ermQ*, the macrolide-lincosamide-streptogramin B resistance gene previously described in *C. perfringens* (Berryman et al., 1994), although this marker was missing in isolates with lower clindamycin resistance MIC values. No β-lactamase marker was identified in the penicillin resistant isolate Cp13-001.

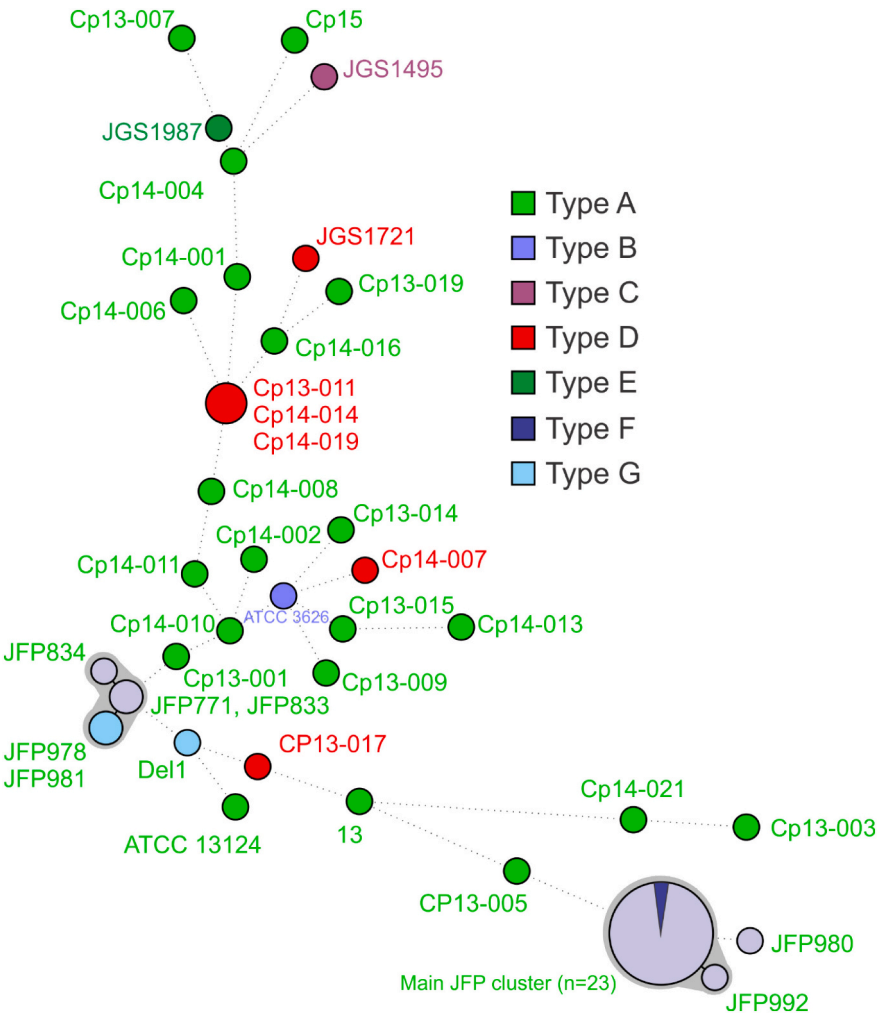

Fig. 1. Minimum spanning tree phylogenetic analysis of *C. perfringens* isolates in this study, together with other strains with publicly available genomes. Phylogeny is based on the standard *C. perfringens* MLST scheme.

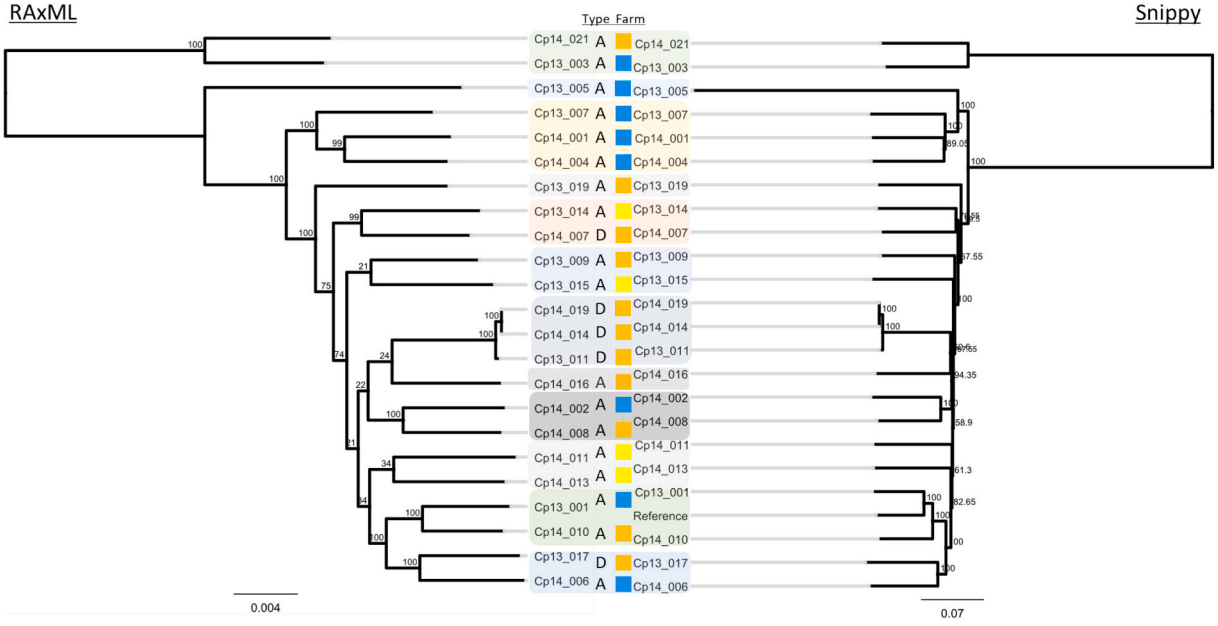

Fig. 2. Phylogenetic analysis of isolates in this study, comparing RAxML analysis of the cohort pangenome with the SNP based approach of Snippy. Farm types: blue, bovine; orange, caprine; yellow, ovine.

Table 2
MIC values of isolates in this study against the antimicrobial panel. Concentrations measured are in white, while those outside the range tested are shaded grey. Where a single thick line is present, concentrations to the left represent sensitive MIC values, whereas those to the right represent MICs designated resistant to that antimicrobial. In the case of two lines, those to the left are sensitive, those between the two lines are intermediate/non-susceptible, and those to the right are considered resistant.

Class	Antimicrobial agent	Farm type	n	% resistant	Antimicrobial concentratrion (mg/L)																														
					<0.002	0.002	0.003	0.004	0.006	0.008	0.012	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32	>32
Ansamycins	Rifampicin	Bovine	8	0	12.5[a]		37.5	37.5	12.5																										
		Caprine	11	0	18.2	9.1	27.3	18.2	27.3																										
		Ovine	4	0			50.0		25.0	25.0																									
Amphenicols	Chlorampenicol	Bovine	8	0																							37.5	12.5	37.5	12.5					
		Caprine	11	0																						18.2	36.3	9.1	27.3	9.1					
		Ovine	4	0																							50.0	25.0	25.0						
Nitroimidazoles	Metronidazole	Bovine	8	0																	25.0	25.0	25.0	25.0											
		Caprine	11	0																18.2	18.2	36.3	27.3												
		Ovine	4	0													25.0		25.0	25.0		25.0													
Tetracyclines	Tetracycline	Bovine	8	50.0											12.5	37.5														12.5	25.0	12.5			
		Caprine	11	0											9.1	36.4	54.5																		
		Ovine	4	0													100.0																		
					<0.016	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32	64	96	128	192	256	>256	
Lyncomicins	Clindamycin	Bovine	8	37.5	12.5						25.0		12.5		12.5																			37.5	
		Caprine	11	0						54.5	27.3	9.1								9.1															
		Ovine	4	0						25.0	25.0								50.0																
					<0.002	0.002	0.004	0.008	0.150	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32														
Penicillins	Penicillin G	Bovine	8	0							12.5	75.0			12.5																				
		Caprine	11	0							27.2	63.7	9.1																						
		Ovine	4	0							25.0	75.0																							
					<0.015	0.015	0.003	0.006	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256														
Macrolides	Erythromycin	Bovine	8	37.5								37.5	25.0				12.5				25.0														
		Caprine	11	0								54.5	36.4	9.1																					
		Ovine	4	0								75.0	25.0																						

[a]Numbers indicate the percentage of isolates, by farm type, with MICs at the indicated antimicrobial concentration.

3.4. Virulence markers

The presence of virulence markers among isolates is shown in Fig. 3. Virulence markers present in all isolates included *colA*, *nanI*, *nanH*, *pfo* and *cpa*. Both *nanJ* and *nagH* were present in all but 2 isolates. Three isolates in this study harboured beta2 (β2) toxin: Cp14-002, Cp13-007 and Cp14-006; all were isolated from bovine farms, with CP14-002 from farm A, and the other 2 isolates from farm B. All 5 type D isolates, which carried *etx*, were from caprine farms. Broadly, isolates could be subdivided into 4 characteristic virulence gene profile groups: I, harbouring *colA*, *nanI*, *nanH*, *nanJ*, *pfo*, *cpa*, and *nagH*; II, carrying *colA*, *nanI*, *nanH*, *pfo*, and *cpa*; III, all aforementioned genes of profile I, with the addition of *cpb2*; and IV, comprising type D isolates and including all virulence genes of profile I, together with *etx*. Profiles I, II and III only included type A isolates.

3.5. Other mobile genetic elements

Plasmids were identified in the majority of isolates (16/23, 70 %); all of these isolates harbouring plasmids contained at least 2 plasmids, with the exception of Cp13-005 and Cp13-009, the former containing a single plasmid of 14,447 bp. All 5 type D isolates contained plasmids carrying the *etx* virulence marker, characteristic of this toxin type. While 4 of these harboured highly homologous plasmids, one of the isolates (Cp13-017) contained additional genes compared with others, and this included 2 polyferrodoxin markers (Fig. 4). Two *etx*-locus variants were identified: plasmid pCp13-017-1 with an IS*231*-IS*1511* locus variant, while the other isolates had a Tn*3*-IS*1511* genotype. Plasmids carrying the *cpb2* virulence marker shower greater variability (Fig. 5). All *etx* and *cpb2* plasmids were conjugal, carrying the TCP transfer locus characteristic of a variety of *C. perfringens* plasmids (Bannam et al., 2006; Li et al., 2013).

The bacitracin resistance locus, ICE*Cp1*, was absent in all farm isolates of this study. Similarly, both the lincomycin resistance element tIS*Cpe8*, and the chloramphenicol resistance transposon Tn*4451*, were also absent from all isolates. The latter aligns with phenotypic screening results, which identified all isolates as sensitive to chloramphenicol.

Analysis of phage insert regions also showed variability across isolates, as shown in Fig. 6. Intact phage were identified in 35 % of the isolates ($n = 8$), whereas 61 % of isolates harboured incomplete prophage insertions. All isolates contained at least 1 phage region in their genomes.

Confirmed 'clustered regularly interspaced short palindromic repeats' (CRISPR) sequences were identified in most isolates ($n = 16$, 70 %). The number of confirmed CRISPR sequence regions varied among individual isolates (Fig. 6), from 0 ($n = 7$ isolates, 30 %) or 1 ($n = 6$, 26 %), up to 9 distinct CRISPRs in a single isolate (Cp14-021). There were 106 unique CRISPR sequences found among the isolates.

4. Discussion

The bacterium *C. perfringens* is of importance to food safety and public health, as well as animal health (Andersson et al., 1995; Sawires and Songer, 2006). As demonstrated in this study, and others, this microorganism may be readily found disseminated throughout dairy farm systems, and as such, good agricultural practice and farm hygiene are important mechanisms to help control this opportunistic pathogen, and safeguard associated safety of dairy products, as well as protect dairy herd health. Since strains may range from commensal or low risk, to high risk of causing acute illness or intoxication in humans and animals, it is crucial to understand the dissemination of related genetic markers among the bacterial population. To this end, we characterized the genomes of isolates associated with bovine, caprine and ovine dairy farms, and examined their phenotypic antimicrobial resistance, to better understand the significance of these dairy-associated isolates to both human and animal health.

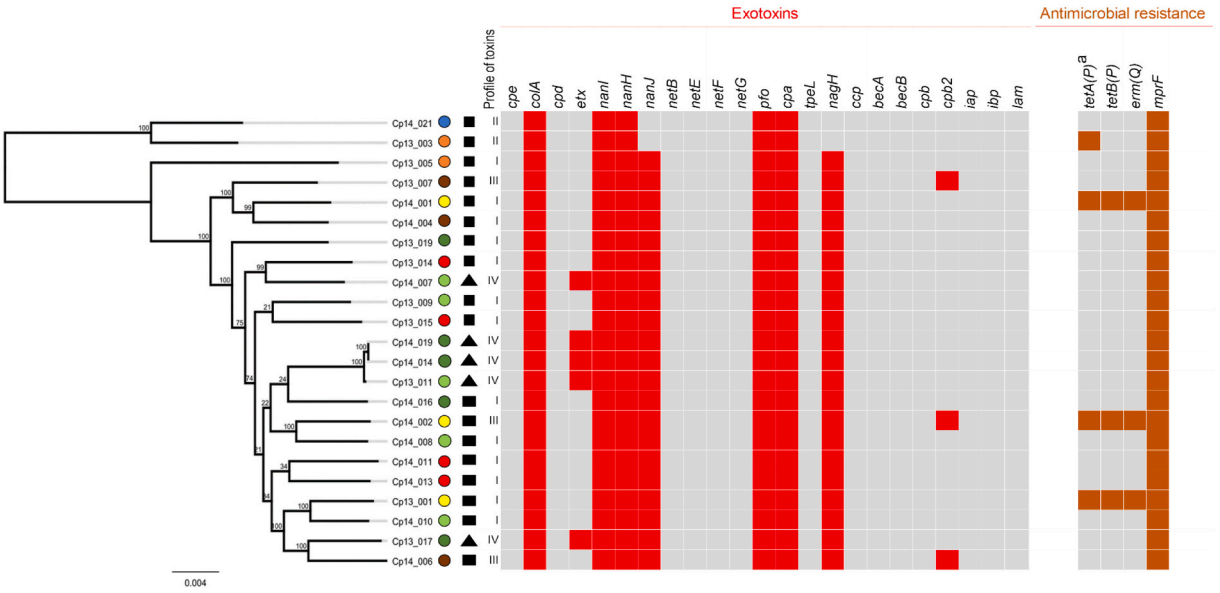

Fig. 3. Phylogenetic analysis and genetic marker heat map. Dendrogram shows a RAxML maximum likelihood clustering analysis, and heat map presents shows presence/absence of selected virulence and antimicrobial resistance (Red, present; grey, absent). Coloured circles indicate farm. Black square, type A; black triangle, type D. 'Profile of toxins' groups isolates by shared virulence gene profile (groups I-IV). Bootstrap values are indicated at branch nodes. [a]Cp13-003 harboured the chromosomal *tetA* variant, *tetA(C)*.

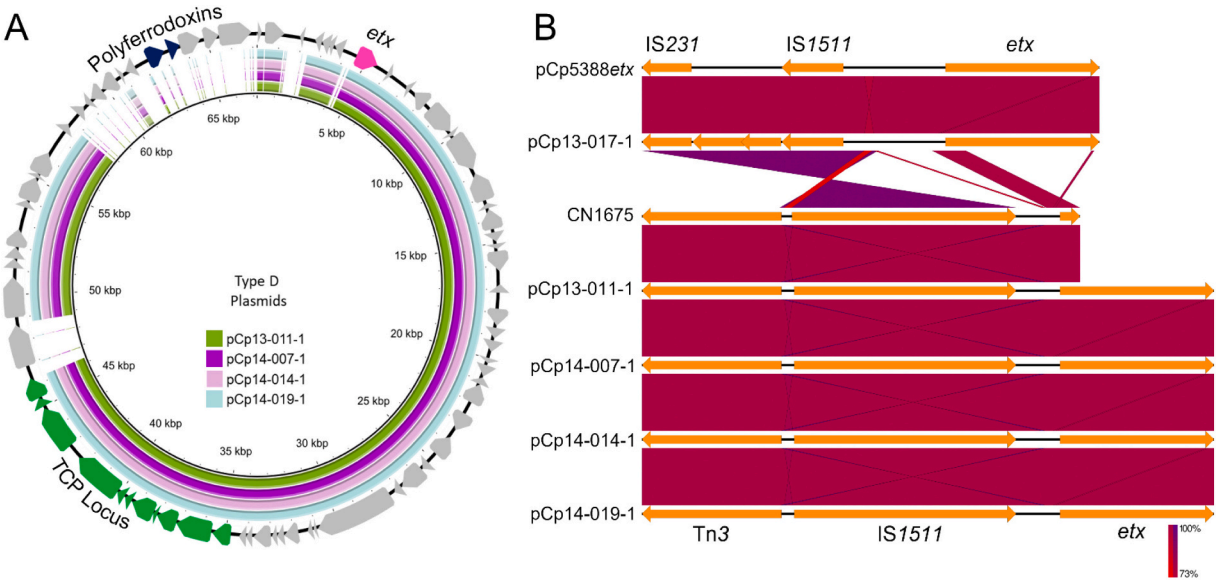

Fig. 4. Comparative analysis of type D isolate plasmids carrying the *etx* epsilon toxin gene. A. BRIG alignment of type D plasmids. The inner ring (black circle) is the reference plasmid, pCp13-007-1. Other plasmids are represented by coloured rings, as indicated in Figure. Outside ring indicates gene coding regions, with selected genes annotated: green, TCP conjugal transfer locus; pink, virulence genes; purple, polyferrodoxin genes. B. Alignment of *etx* gene locus, showing two variants: the IS*231*-related/IS1511-like transposase variant (pCp5388*etx* and pCp13-017-1); and the Tn*3*-like IS*1511* variant (CN1675, pCp13-011-1, pCp14-007-1, pCp14-014-1, and pCp14-019-1).

As reported in previous ruminant-associated and dairy farm studies, type A was the most common type identified among the *C. perfringens* population (Athira et al., 2018; Fohler et al., 2016; Forti et al., 2020; Geier et al., 2021). Analysis of the population structure revealed a diverse population, comprising largely novel sequence types, with large genetic variation across the pangenome. The type D isolates showed greater genetic similarities than that of the type A isolates in this study. This was evident from the MLST minimum spanning tree, in which 3 type D isolates shared a ST, as well as the pangenome analysis, where the type D isolates shared a core genome of 56.7 % of the pangenome, compared with just 23.0 % core genome considering type A isolates. It should be noted, however, that type D group comprised a smaller number of isolates ($n = 5$); nonetheless, this closer sequence homology was also supported by the phylogenetic analyses, clustering 3 type D isolates closely (Cp13-011, Cp14-014 and Cp14-019). Of these 3 type D isolates, 2 came from a single caprine farm (Cap2), with the remaining isolate from another caprine farm (Cap1). This may suggest a disseminated clone on farm Cap2, however, given the distinct geographical locations of caprine farms Cap2 and Cap1, this type D genotype variant may be circulating more widely across the state of Victoria. More generally, however, the phylogenetic analyses of these isolates demonstrated high diversity among the population. Although some clustering

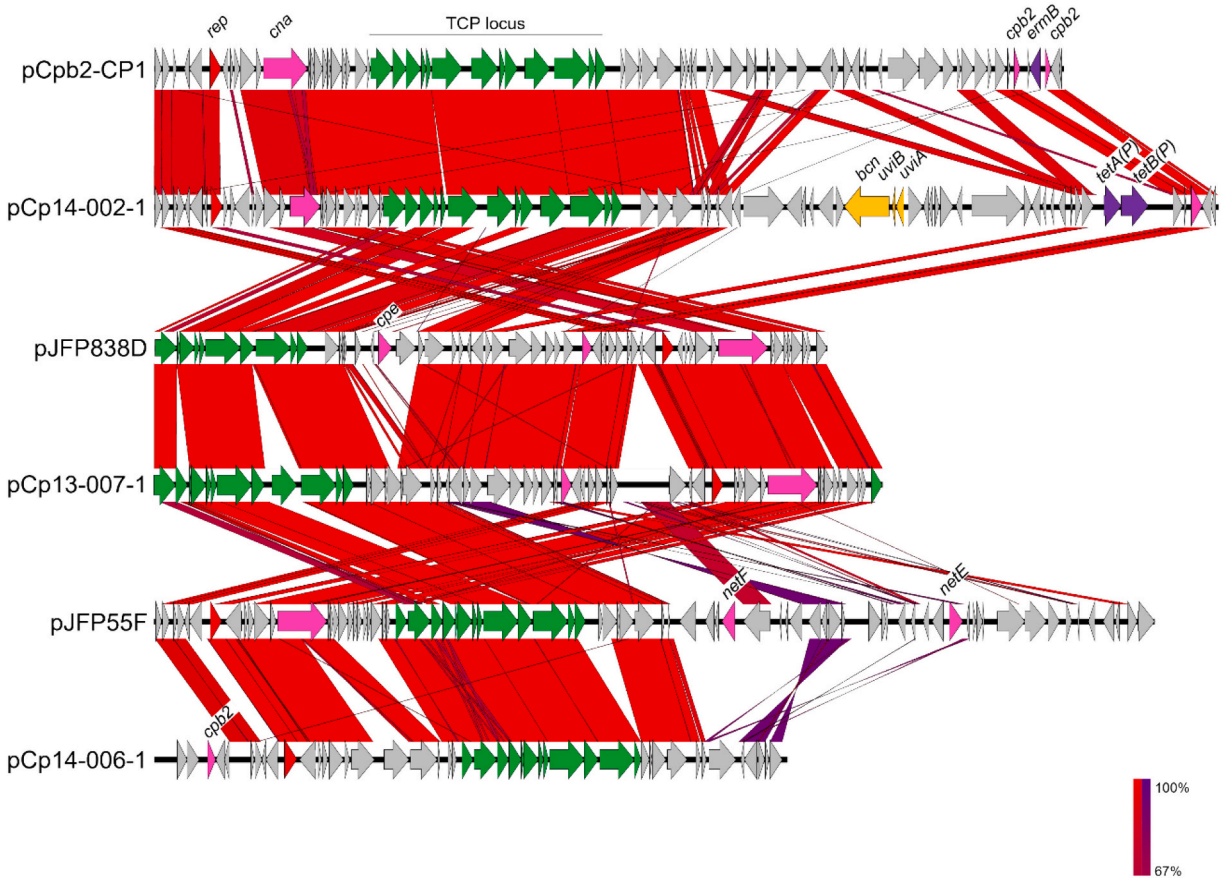

Fig. 5. Sequences of *cpb2*-carrying plasmids, including 3 isolates from this study (pCp14-002-1, pCp13-007-1 and pCp14-006-1), together with an additional 3 reference sequences sharing high BLAST similarity scores to one of the 3 (pCpb2-CP1, pJFP838D and pJFP55F, respectively). Green arrows, TCP conjugal transfer locus; red, replication gene; pink, virulence genes; orange, stress resistance; purple, antimicrobial resistance.

was observed by subsets of isolates belonging to an individual toxin type, they could not be grouped exclusively by toxin type. This supports the observation that, with many of the genetic determinants grouping *C. perfringens* into toxin types carried on mobile genetic elements, horizontal gene transfer is an important driver of expansion of individual toxin type populations.

In order to provide a more robust analysis of the population structure, the phylogeny was solved using two different approaches: a RAxML maximum likelihood analysis, and a SNP subtyping analysis using snippy. As can be seen in Fig. 2, there was notable agreement between dendrograms generated with both approaches. This strengthens the confidence in the validity of the phylogenetic clustering of isolates in this study, and further supports that, with the exception of a more closely related clade including 3 type D isolates (Cp13-011, Cp14-014, and Cp14-019), the population was generally diverse and not clonal. Two type A outliers clustered away from the other isolates: Cp13-003 and Cp14-021. This was also supported in the MLST analysis (Fig. 1). Some notable features of these isolates relative to others in this study, include the chromosomal *tetA(C)* tetracycline resistance marker unique to Cp13-003, and the high number of confirmed CRISPR sequence regions in Cp14-021 (9 regions, more than any other in this study). Both these isolates also lacked virulence factors present in other isolates, such as *nanJ* and *nagH*.

Analysis of virulence genes showed 4 characteristic genotypes, with groups sharing a core set of markers, but distinguished by presence/absence of the markers *etx*, *cpb2*, or *nanJ/nagH* (Fig. 3). All groups lacked *cpe*, which codes for *Clostridium perfringens* enterotoxin (CPE), characteristic of *C. perfringens* food poisoning. This suggests that isolates associated with dairy farms in this study do not show widespread distribution of this marker, and may be lower risk for causing gastrointestinal illness in associated contaminated dairy products. Indeed, previous studies support this observation that dairy farms are not significant sources of *cpe*-positive isolates, with Fohler et al. (2016) finding similar low incidence on dairy farms in Germany (0.8 % of type A isolates carried *cpe*), and Geier et al. (2021) finding low incidence in ruminant-associated isolates (2.9 %), compared with high incidence of *cpe* carriage among canine, equine and food isolates (94.1 %, 93.8 %, and 86.7 %, respectively). Two toxins of importance to animal health in particular, were identified among isolates in this study: the epsilon toxin ETX and beta2 toxin. The ETX toxin was carried on a conjugal plasmid, with two distinct plasmid variants, as illustrated in Fig. 4. These plasmids shared a plasmid backbone typical of other type D plasmids; however, all lacked the *cpb2* marker, which can be found on some type D plasmid variants such as pCP8533etx (Miyamoto et al., 2008). The potent ETX toxin is associated with severe disease in ruminants, particularly smaller ruminant such as caprine and ovine species (Lebrun et al., 2010). Most potent of the *C. perfringens* toxins, this pore-forming toxin can lead to acute diseases such as exnterotoxemia, with rapid onset and mortality (Popoff, 2011). In this study, all 5 type D isolates were from samples taken at farms keeping caprine herds, and in one case from a feed sample. This is cause for concern, since these animals are high risk of illness from type D enterotoxemia. This emphasizes the importance of monitoring for, and controlling, *C. perfringens* in dairy farm niches. Furthermore, given *etx* and *cpb2* were plasmid-borne, there is potential for wider dissemination of these virulence markers throughout the farm environment, again highlighting the importance of controlling these bacterial pathogens.

A recent study by Geier et al. (2021) provided a comparative

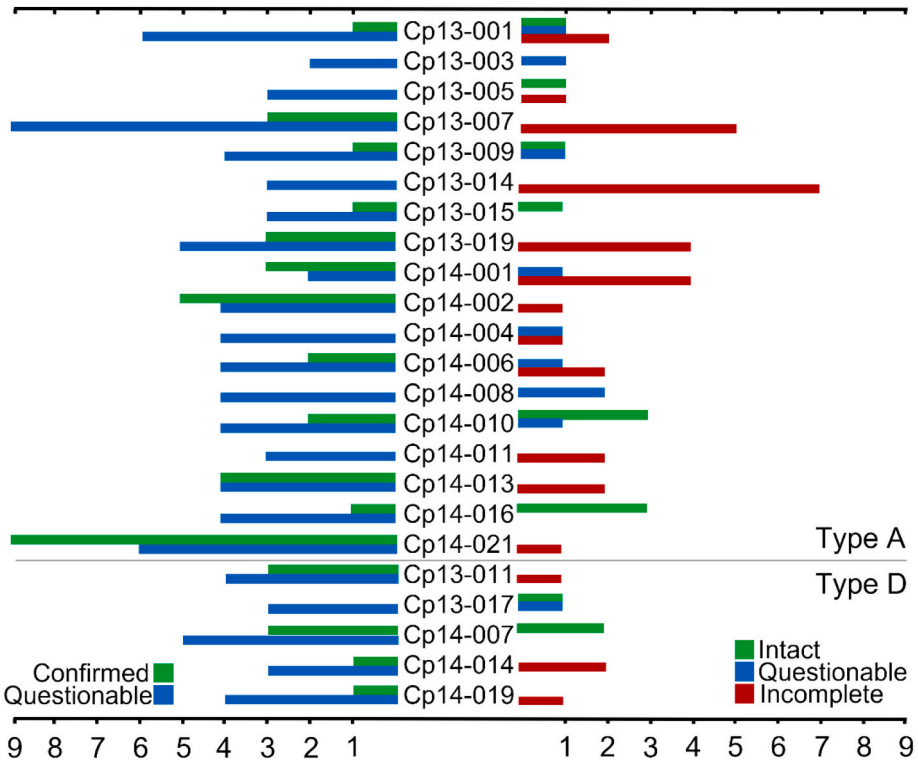

Fig. 6. Number of CRISPR and phage inserts identified among isolates in this study. CRISPR numbers in each isolate are indicated by the bars to the left, while phage inserts are indicated by the bars to the right.

genomic characterization of *C. perfringens* isolates from diverse sources, to examine population structure and trends in virulence marker carriage. Among the 34 ruminant isolates, similar virulence profiles to isolates from this study were noted. This was characterized by absence of enterotoxin and *netB* genes (both our study, and theirs, did not identify *netB*, *netE*, *netF*, or *netG*), while all isolates carried *colA*, *nanI* and *nanH*. Similar low carriage rates of *etx* and *cpb2* were supported by both studies. Interestingly, all ruminant isolates in Geier et al. (2021) harboured *nagH*, while Cp13-003 and Cp14-021, the two outliers in our phylogenetic analyses, lacked this virulence marker. This supports the case that these are atypical of other type A ruminant-associated isolates. Both studies supported a high overall genetic diversity among rumiant-associated *C. perfringens*.The plasmid-borne β2 toxin is encoded by *cpb2*, and is implicated in enteric disease in both humans and other animals, notably in its demonstrated cytotoxicity to porcine intestinal epithelial cells (Luo et al., 2020; van Asten et al., 2010). In our study, this toxin was identified 3 isolates ($n = 3$), and all were toxin type A. While all 3 plasmids harboured the characteristic TCP conjugal transfer locus, sequence comparison showed heterogenous regions in each plasmid. Previously described plasmids showing high similarity scores to those identified in this study are shown in Fig. 5. However, plasmids in this study lacked other virulence markers found on their closest matched pairs: pCp13-007-1 was closely related to pJFP838D, however lacked the *cpe* marker of the latter; similarly, both *netE* and *netF*, virulence markers carried on pJFP55F, were absent in pCp14-006-1 from this study.

Common antimicrobial treatments of *C. perfringens* infection in humans have included the use of penicillin, clindamycin, tetracycline, chloramphenicol, or metronidazole, and these were screened in this study (Bryant and Stevens, 2010). Interestingly, antibiotic treatment has also been implicated as a pre-disposing factor in disease etiology, and may induce overproduction of toxins (Allaart et al., 2013; Vilei et al., 2005). Resistance to these key antibiotics varied among isolates from this study; no resistance was observed to chloramphenicol, metronidazole, or rifampicin, suggesting these to be effective drugs of choice against isolates characteristic of farms in this study. This aligns with other studies, which also found relatively low, or no, resistance among *C. perfringens* isolates from various sources (Table 3). Although clindamycin resistance was observed in 30 % of isolates in this study, similarly high (or higher) levels have been reported from other studies in various global regions. Interestingly, isolates from this study phenotypically separated into 'less resistant' isolates with a lower MIC (between 0.38 and 2 mg/L), and higher levels of resistance (>256 mg/L). The latter group all carried the *erm(Q)* resistance marker, which is associated to lincosamide resistance and likely explains the high tolerance observed in those isolates (Berryman et al., 1994; Leclercq and Courvalin, 1991). The presence of this resistance marker is also associated with resistance to macrolides and streptogramin B, and explains the phenotypic resistance to erythromycin these 3 isolates also showed. Other studies have also noted relatively higher prevalence of resistance to clindamycin and erythromycin among isolate cohorts, and collectively this suggests these may be sub-optimal choices in treatment where the antibiogram is unknown (Anju et al., 2021; Li et al., 2020; Slavić et al., 2011).

In animals, treatment of type D-related infection is problematic. Due to the rapid onset of severe enterotoxemia once symptoms prevent, treatment is difficult as mortality can occur within days, characterized by sudden death syndrome (Uzal and Songer, 2008). As such, vaccination systems are the primary approach for disease protection. However, reports have indicated antibiotic treatment of animals may impact severity of subsequent *C. perfringens* disease; Vilei et al. (2005) demonstrated gentamicin or streptomycin induced the expression of *cpb2*, and suggest such treatment may lead to severe colitis in horses based on analysis of clinical samples. This may be cause for consideration for farms in this study, given the detection of *cpb2* among the type A isolates; particularly, if there is intestinal carriage of such a strain during antimicrobial-induced dysbiosis (one of the type D isolates, Cp13-011, was recovered from a feed sample, suggesting a direct route of exposure of animals, and possible enteric transit). A previous report on postpartum mortality of bovines due to type A *C. perfringens* also noted that physiological stressors and imbalances in the intestinal flora may

Table 3
Reports of antimicrobial resistance prevalence among *C. perfringens* isolates from various sources, from studies between 1977 and 2022. Values are percentage of resistant isolates among those tested. Where no value is shown, data for this antibiotic was not presented in the study. 'Year sampled' refers to the most recent year *C. perfringens* was isolated in that study; an underlined year means this date was not specified in the study.

Year sampled	Country	C	DA	E	MTZ	P	RD	TE	Reference
2021	India	–[a]	–	40	–	16	17.3	26.7	Anju et al. (2021)
2020	Pakistan	–	50	60	0	0	–	95	Haider et al. (2022)
2018	China	–	26.9	49.1	–	23.1	–	20.1	Li et al. (2020)
2015	Spain	–	0	0.9	4.6	2.8	0	7.3	Álvarez-Pérez et al. (2017)
2014	Spain	–	0	0	5.7	0	0	–	Álvarez-Pérez et al. (2016)
2014	*Australia*	*0*	*30.4*	*13*	*0*	*4.3*	*0*	*4.3*	*This study*
2010	Côte d'Ivoire	20	–	45	–	5	–	50	Kouassi et al. (2014)
2009	USA	0	66.7	–	4.8	12.9	–	–	Lawhon et al. (2013)
2005	Canada	–	11.6	10.2	0.4	–	–	75.6	Slavić et al. (2011)
2004	Thailand	3	–	–	9.5	9	–	56.2	Tansuphasiri et al. (2005)
2001	USA	–	–	6.1	0.8	–	–	21.4	Marks and Kather (2003)
1997	South Africa	0	14	–	0	0	–	–	Lubbe et al. (1999)
1977	USA	0	≥25	–	–	0	2	19	Schwartzman et al. (1977)
1974	USA	–	≥52	–	≥44	≥4	–	–	Staneck and Washington (1974)

C, chloramphenicol; DA, clindamycin; E, erythromycin; MTZ, metronidazole; P, penicillin G; RD, rifampicin; TE, tetracycline.
[a] -, not tested in study.

contribute to severe infection and death in the animal, again highlighting the potential risks of intestinal carriage of *C. perfringens* during episodes of gut microbiome dysbiosis (Lu et al., 2022).

Tetracycline resistance is among the most commonly reported from studies examining resistance phenotypes among *C. perfringens* isolates, as indicated in Table 3. Comparatively, resistance was lower in this study, with only a single isolate showing a resistance phenotype (3 %) and with 17.4 % ($n = 4$) non-susceptible, based on CLSI S/I/R guidelines. Tetracycline non-susceptibility was associated with the presence of *tetA*, with or without *tetB*. In the case of three isolates, *tetA(P)*- and *tetB(P)*-mediated resistance to tetracycline was harboured on three plasmid variants: for Cp13-001, the resistance plasmid pCp13-001-1 was a homolog of pCW3 (99.94 % identity; Supplementary Fig. 2), a well-characterized conjugal resistance plasmid (Bannam et al., 2006). In isolate Cp14-001, the tetracycline resistance plasmid pCp14-001-1 was also closely related to pCW3/pCp13-001-1, with two additional CDSs, a *tnpA* homolog and resolvase, present. The last tetracycline resistance plasmid from Cp14-002 was a larger plasmid also carrying the *cpb2* gene. Interestingly, the pCW3 plasmid was originally described in 1978, in a strain collected from an abscessed appendix of a human patient (Rood et al., 1978). This supports the observation that pCW3-like conjugal plasmids are globally distributed through diverse niches, associated with humans, animals, and the wider environment (Abraham et al., 1985). In the case of the remaining isolate showing a non-susceptible tetracycline phenotype, Cp13-003, the *tetA(C)* resistance marker was chromosomally located. Based on comparisons with other strains, this appears to be located in a region showing phage-related proteins, suggesting this may be an artefact of previous phage-related recombination (Supplementary Fig. 3). The region was similar to that of LLY_N11, a strain isolated from a chicken intestine; while in the ATCC 13124 Type strain, an approximately 12 kb region containing the *tetA(C)* marker in the other 2 isolates, is absent.

A single isolate, Cp13-001, showed higher tolerance to penicillin, with an MIC of 1 μg/mL indicating resistance based on EUCAST guidelines (or intermediate as per CLSI breakpoints). Penicillin resistance has been previously described in *C. perfringens*-associated clinical infection (Mishra et al., 2016), and beta-lactamase genes (*bla* genes) have previously been identified in resistant isolates (Ali and Islam, 2021). We did not identify any *bla* genes among isolates in this study, including Cp13-001. In the absence of β-lactamase, mutations in the penicillin binding proteins (PBPs), enzymes involved in cell wall peptidoglycan biosynthesis, may alter the capacity of β-lactam antibiotics to bind to PBPs (Macheboeuf et al., 2008; Park et al., 2020). In *C. perfringens*, such a mutation in PBP1 has been identified, comprising a Gly871Cys amino acid substitution immediately upstream of the KTGT motif; this motif is a target involved in binding of the antibiotic (Park et al., 2020). Analysis of the Cp13-001 PBP1 sequence showed that, similarly to all other isolates, this mutation was not present (all isolates harboured the wild type GKTKT sequence at residues 871–875). However, a unique substitution in Cp13-001 when compared with other isolates in this study (including the penicillin-sensitive Type strain ATCC 13124), was noted 3 residues upstream of this (V868A); a possible role for this in the higher penicillin tolerance of Cp13-001 requires further study.

Plasmids were identified in the majority of isolates in this study (70 %), including isolates from 6 of the 7 farms, suggesting widespread dissemination of these mobile genetic elements among the *C. perfringens*. Interestingly, all but one of these plasmid-bearing isolates harboured a conjugative plasmid, mobilised by the globally disseminated TCP conjugal transfer locus (Bannam et al., 2006). The high prevalence of conjugative plasmids supports the high frequency of plasmid carriage. The more recently described PCP conjugal transfer locus was only identified in a single isolate, Cp13-007, suggesting this class of conjugal plasmid is less disseminated among *C. perfringens* isolates across the dairy farms (Watts et al., 2019). Isolate Cp13-007 contained both TCP- and PCP-mobilised plasmids, indicating these can co-exist within the same isolate. Isolate Cp13-005 was the only one to contain a non-conjugal plasmid. The pCp13-005-1 plasmid was a smaller plasmid of 14,447 bp (Supplementary Fig. 4). Analysis of the markers carried on this plasmid identified features such as an abortive infection protein, a HlyD family secretion protein, and a CAAX amino terminal protease family protein. Such proteins have been associated with roles in toxin/antitoxin or bacteriocin systems, suggesting this plasmid may have a role in bacterial competition (Kjos et al., 2010; Pei et al., 2011; Pimenta et al., 2005). Further work is required to explore such a potential role.

The CRISPR systems of bacteria have been associated with resistance to phage infection (Bolotin et al., 2005). In this study, we identified 136 unique CRISPR regions (confirmed or questionable, as designated by CRISPRFinder); all isolates were found to contain at least 2 questionable CRISPR regions, and 70 % of isolates had confirmed CRISPRs ($n = 16$). Of these, we found 17 unique CRISPRs found in at least 2 isolates (Supplementary Fig. 5); taken together, this suggests CRISPR systems are common in *C. perfringens* isolates, with particular genotypes more widely disseminated throughout the bacterial population.

This study provides insights into the molecular ecology and genotypic traits of *C. perfringens* isolates from dairy farms. Population analysis showed *C. perfringens* has a diverse phylogeny, which does not appear to segregate strongly by toxin type. Twenty novel STs were described, across both type A and D toxin types. Most isolates harboured plasmids, which mobilised virulence and AMR markers. Although antimicrobial resistance was detected, levels were generally comparable, or

lower, to levels reported in other global regions. The presence of type D isolates disseminated across caprine farms highlights the utility of surveillance of *C. perfringens* on these farms, particularly in feed samples, to protect animal health. As has been previously shown, factors leading to gut dysbiosis can facilitate development of severe infections by *C. perfringens* in ruminants, thus the application of such surveillance, including new diagnostic approaches for herd detection, could help mitigate these negative impacts to herd health.

Declaration of competing interest

No conflict of interest declared.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2022.109933.

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